Expression of Cyclin D1 and P16 in Esophageal Squamous Cell Carcinoma

Biswajit Dey[1*], Vandana Raphael[1], Yookarin Khonglah[1], Kyrshanlang GiriLynrah[1]

1. North Eastern Indira Gandhi Regional Institute of Health and Medical Sciences, India

* **Corresponding Author:**
Biswajit Dey, MD
Department of Pathology, NEIGRIHMS, Shillong- 793018, Meghalaya, India
Tel: + 91 9655166544
Fax:+ 91 03642538003
Email: drbish25@rediffmail.com
Received: 06 Apr. 2015
Accepted: 07 Jul.2015

ABSTRACT

BACKGROUND

Esophageal squamous cell carcinoma (ESCC) is one of the lethal cancers with a high incidence rate in Asia. Many genes including cyclin D1 and p16 play important role in its carcinogenesis. We aimed to analyze the expressions of cyclin D1 and p16 with the various clinicopathological characteristics of ESCC.

METHODS

We examined 30 biopsy samples of ESCC for cyclin D1 and p16 protein expressions using immunohistochemistry. Immunointensity was classified as no immunostaining (-), weakly immunostaining (+), weak immunostaining (++) and strongly positive immunostaining (+++).

RESULTS

Out of the 30 cases, positive expression of cyclin D1 was detected in 26 cases (86.7%). The percentage of tumors with invasion to the adventitia (88.2%), lymph node metastasis (87.5%), and tumors which were poorly differentiated (92.9%) were higher in cyclin D1 positive tumors than in the cyclin D1 negative tumors. However no significant association was found between cyclin D1 expression and the different clinicopathological parameters.There were 22 cases of ESCC (73.3 %) which showed negativity for p16. The percentage of tumors with invasion to the adventitia (82.4%) and poorly differentiated tumors (92.9%) were higher in the p16 negative tumors than in the p16 positive tumors. There was significant association between the histological grade and p16 expression (p=0.012). However, there were no significant association with regard to site, size and lymph node status of the tumors and p16 expression.

CONCLUSION

The study shows that alterations of cyclin D1 and p16 play an important role in ESCC. Loss of p16 expression was associated with poor differentiation.

KEYWORDS

Esophageal squamous cell carcinoma; Immunohistochemistry; Cyclin D1; P16

Please cite this paper as:
Dey B, Raphael V, Khonglah Y, GiriLynrah K. Expression of Cyclin D1 and P16 in Esophageal Squamous Cell Carcinoma. *Middle East J Dig Dis* 2015;7:222-7.

INTRODUCTION

Esophageal cancer has become the sixth leading cause of death from cancer worldwide.[1] More than 90% of esophageal cancers are either squamous cell carcinoma or adenocarcinoma.[2] Esophageal squamous cell carcinoma (ESCC) is the predominant histological subtype worldwide with high incidence rate in Asia.[3] To the best of our knowledge, there is no data available regarding the incidence or epidemiology of ESCC in the study area. However, in the neighboring state of Assam in the north-east region of India, it is the leading cause of cancer in men and ranks second in women.[4]

Various genetic alterations occur during the process of esophageal carcinogenesis. Although tremendous progress has been made in surgery and adjuvant chemoradiotherapy, prognosis of these patients remains poor. A detailed search into these genetic alterations can provide crucial clues to discover novel biomarkers for improving diagnosis and guiding targeted therapy.[5]

Among the various genetic alterations studied as putative biomarkers in ESCC, cyclin D1 and p16 have an important role in ESCC. Gene amplification and over-expression of cyclin D1 have been frequently demonstrated in ESCC. Amplification of cyclin D1 results in growth advantage for tumor cells and enhances tumorigenesis. p16 is involved in the pathogenesis of esophageal cancer by influencing the cyclin kinase inhibitor cascade and DNA mismatch repair processes.[6]

In the present study, expressions of cyclin D1 and p16 in ESCC were evaluated and their association with various clinico-pathological parameters was evaluated to study their role as prognostic markers in ESCC.

MATERIALS AND METHODS

The study was conducted in a group of 30 consecutive patients with primary ESCC without any prior history of chemotherapy or radiotherapy. Patients with inadequate biopsy or inadequate tissue for immunohistochemistry were excluded from the study. The diagnosis was based on endoscopic examination and histopathological examination of the esophageal endoscopic biopsies and/or resection biopsies of the patients admitted in Neigrihms hospital from January 2011 to January 2012.Union International Cancer TNM classification guidelines were used for staging.[7] The study was approved by the Research and Ethics Committee, Neigrihms, Shillong.

Tumors were histologically verified as ESCC and sub-typed based on the grade of differentiation as well differentiated (G1), moderately differentiated (G2) or poorly differentiated (G3). Assessment of cyclin D1 and p16 were done immunohistochemically by Horse Radish Peroxidase (HRP) method. The paraffin embedded sections of the tumor were stained by a monoclonal antibody raised against cyclin D1 (Biogenex, CA, USA) and p16 (Biogenex, CA, USA).

Moreover, 4-5 um sections were placed from paraffin blocks over the poly L-lysine coated slides. The sections were baked by keeping the slides in a hot plate at 60 ºC for one hour and deparafinized by using xylene 1 and xylene 2 for 10 minutes each. Rehydration was done in graded alcohol (absolute, 90%, 70%) for 5 minutes each. They were placed in 10 mmol/L citrate buffer (pH=6.0) to unmask the epitopes. After microwave antigen retrieval (5 min, 450 W; 5 min, 600 W), the sections were allowed to cool down to room temperature (approximately 20 min). The slides were washed with phosphate buffer solution for three times with 1 minute interval. After blocking the non-specific protein binding sites with serum free protein block for 10 minutes at room temperature, the slides were again washed with phosphate buffer solution for three times with 1 minute interval. Power block was added on the sections for 10 minutes at room temperature. Primary antibody was then added and the slides were incubated in humidity chamber for 60 minutes at room temperature and washed with phosphate buffer solution thrice with 1 minute interval. After addition of super enhancer for 30 minutes at room temperature and washing with phosphate buffer solution thrice with 1 minute interval, polymer HRP (secondary antibody) was added and incubated in

humidity chamber for 30 minutes at room temperature. The slides were washed with phosphate buffer solution thrice with 1 minute interval and 3,3 Diaminobenzidine chromogen was added and incubated for 5 to 10 minutes at room temperature. The slides were then washed in distilled water and counter stained with Mayer's hematoxylin for 1 to 2 minutes. The slides were put in running tap water for 5 minutes and dehydrated in graded concentration of alcohol (70%, 90%, absolute) for 1 minute each. Thereafter, the slides were mounted with distyrenephthalate xylene.

Each slide was examined under a light microscope without the knowledge of the patient's other data. A minimum of 100 neoplastic cells were examined. According to the intensity of staining of neoplastic cells and the percentage of positive cells, the results of immunostaining in tissues were scored. Immunoreactivity was classified into the following three categories based on the percentage of tumor cells showing nuclear reactivity: less than 10%, 10-50% and more than 50%. Immunointensity was classified as no immunostaining (-), weakly immunostaining (+), weak immunostaining (++) and strongly positive immunostaining (+++). For cyclin D1 staining, if strong nuclear staining was recognized in more than 10% of the tumor cells, the specimen was considered to be positive. If strong nuclear staining was seen in less than 10% of the tumor cells, the specimen was judged to be negative. Positivity for p16 staining was considered if more than 80% of the tumor cells showed strong nuclear staining. If less than 80% of the tumor cells showed nuclear staining, the specimen was considered to be negative.[8]

Normal esophageal epithelia, which represented a positive control, were included in each run and the negative control section was carried out by omitting the primary antibody. We also considered the adjacent non-neoplastic squamous epithelia to compare the positive staining in tumors. In cyclin D1 immunostaining, adequate nuclear staining was observed in the benign controls in the normal epithelia in all cases, which represented a positive control. The benign controls showed p16 expression in less than 20% of cells with weak intensity mainly confined to the basal epithelial cells.

Association between cyclin D1 and p16 expression with clinicopathological parameters were evaluated by Chi-Square (x^2) test and Fischer's exact test. Probability (p) less than 0.05 was considered statistically significant.

RESULTS

A total of 30 patients with histologically confirmed squamous cell carcinoma of the esophagus were included in the study. There were 20 men and 10 women. The age of the patients ranged from 36 years to 79 years with a mean of 53.2 years. Histological grading of the 30 patients was as follows: 2 patients were well differentiated ESCC (G1), 14 patients were moderately differentiated ESCC (G2) and 14 patients were poorly differentiated ESCC (G3). There were 7 cases in stage I, 8 in stage II, 11 in stage III, and 4 in stage IV. In all the 30 patients of ESCC, the association of p16 and cyclin D1 expression and the clinicopathological parameters like tumor

site, tumor size, tumor invasion to adventitia, lymph node metastasis and histological grade of tumors were analyzed.

Out of the 30 patients, positive expression of cyclin D1was detected in 26 (86.7%) patients and the remaining 4 patients were cyclin D1 negative (13.3%). The percentage of tumors with invasion to the adventitia (88.2%), lymph node metastasis (87.5%), and tumors which were poorly differentiated (92.9%) were higher in the cyclin D1 positive tumors than in the cyclin D1 negative tumors. However, no significant association was found between cyclin D1 expression and the different clinicopathological parameters (Table 1).

We found that 22 (73.3 %) patients had ESCC which showed negativity for p16. The remaining 8 (26.7%) patients were positive for p16. The percentage of tumors with invasion to the adventitia (82.4%) and poorly differentiated tumors (92.9%) were higher in the p16 negative tumors than in the p16 positive tumors. There was significant associa-

Table 1: **Distribution of variables in 30 cases of ESCC by cyclin D1 status**

		Total number (n= 30)	Cyclin D1 negative (n= 4)	Cyclin D1 positive (n= 26)	*p* value
Tumor size (cm)	≤ 3 cm	15	1 (6.7%)	14 (93.3%)	0.597
	> 3 cm	15	3 (20%)	12 (80%)	
Tumor site	Upper 1/3rd (C15.3)	0	0	0	0.522
	Middle 1/3rd (C15.4)	12	1 (8.3%)	11 (91.7%)	
	Lower 1/3rd (C15.5)	15	2 (13.3%)	13 (86.7%)	
	Overlapping lesion (C15.8)	3	1 (33.3%)	2 (66.7%)	
Tumor invasion to adventitia	No	13	2 (15.4%)	11 (84.6%)	1.000
	Yes	17	2 (11.8%)	15 (88.2%)	
Lymph node metastasis	Absent	14	2 (14.3%)	12 (85.7%)	1.000
	Present	16	2 (12.5%)	14 (87.5%)	
Histologic type of tumor	Well differentiated(G1)	2	1 (50%)	1 (50%)	0.246
	Moderately differentiated(G2)	14	2 (14.3%)	12 (85.7%)	
	Poorly differentiated(G3)	14	1 (7.1%)	13 (92.9%)	

Table 2: **Distribution of variables in 30 cases of ESCC by p16 status**

		Total number (n= 30)	p16 negative (n= 22)	p16 positive (n= 8)	*p* value
Tumor size (cm)	≤ 3 cm	15	11 (73.3%)	4 (26.7%)	1.318
	> 3 cm	15	11 (73.3%)	4 (26.7%)	
Tumor site	Upper 1/3rd (C15.3)	0	0	0	0.711
	Middle 1/3rd (C15.4)	12	8 (66.7%)	4 (33.3%)	
	Lower 1/3rd (C15.5)	15	12 (80%)	3 (20%)	
	Overlapping lesion (C15.8)	3	2 (66.7%)	1 (33.3%)	
Tumor invasion to adventitia	No	13	8 (61.5%)	5 (38.5%)	0.242
	Yes	17	14 (82.4%)	3 (17.6%)	
Lymph node metastasis	Absent	14	11 (78.6%)	3 (21.4%)	0.688
	Present	16	11 (68.8%)	5 (31.2%)	
Histologic type of tumor	Well differentiated(G1)	2	0	2 (100%)	0.012
	Moderately differentiated(G2)	14	9 (64.3%)	5 (35.7%)	
	Poorly differentiated(G3)	14	13 (92.9%)	1 (7.1%)	

tion between the histologic grade and p16 expression (p=0.012). However, there were no significant association with regard to site, size and lymph node status of the tumors and p16 expression (Table 2).

DISCUSSION

Cyclin D1 gene encodes a protein that complexes with a cyclin dependent protein kinase (CDK) to phosphorylate pRb protein and promote cell's advancement from the G1 phase to the S phase. Overexpression of cyclin D1 is thought to override the G1 checkpoint, driving tumor cell proliferation.[9] Amplification of cyclin D1 results in growth advantage for tumor cells and enhances tumorigenesis.[6] In ESCC, cyclin D1 overexpression plays an important role in cell transformation.[9] Studies on ESCC have shown that cyclin Dl overexpression, evaluated by immunohistochemical staining, results from cyclin DI amplification.[8]

In the present study, positive expression of cyclin D1 was detected in 86.7% of the patients. The percentage was higher than in other high incidence areas like Japan, South Africa, China and other regions of India. In a study done in Japan, the re-

searchers found 25% cyclin D1 overexpression.[8] Similarly in South Africa, Chetty and co-workers demonstrated 29% of ESCC immunopositivity for cyclin D1 and in China, Lin and colleagues demonstrated 56.5% cyclin D1 immunopositivity.[10,11] In a study done in India, 67% immunopositivity for cyclin D1 was shown.[12] These differences in results reflect geographical and epidemiological variations.

Local dietary habits consist of rice along with fish or meat preparations. Moreover hot chilli, smoked meat and hot tea are quite popular. In a study done by Phukan and colleagues in the north-east region of India, it was found that consumption of very spicy foods, hot foods and beverages, a diet containing high amounts of chilli and leftover food was positively associated with the risk of esophageal cancer.[13] Moreover tobacco smoking, betel quid chewing and alcohol consumption are the major known risk factors for esophageal cancer.[14] Betel quid chewing, a common habit in south-east Asia has been found to increase the risk of developing ESCC by 4.7-13.3 fold, although other exogenous risk factors may also be involved.[14] The north-east Indian variety of betel nut, locally known as 'kwai', is raw, wet and consumed unprocessed with betel-leaf and slaked lime and contains higher alkaloids, polyphenol and tannins, which has been found to be genotoxic.[15] This assumes importance since using fermented areca nuts with any form of tobacco is a common habit in the area of the study and is a potential risk factor of ESCC in this region.

Association of cyclin D1 expression with clinicopathological parameters were analyzed in the present study. The percentage of tumors with invasion to the adventitia (88.2%), lymph node metastasis (87.5%) and tumors which were poorly differentiated (92.9%) were higher in the cyclin D1 positive tumors than in the cyclin D1 negative tumors. However, there were no significant association with cyclin D1 expression with regard to site, size, tumor invasion, lymph node status and histological grade of the tumors. Similar results were obtained in other studies.[8,11] Takeuchi and colleagues observed that the percentage of cyclin D1 positive tumors was higher in tumors with invasion to the adventitia and lymph node metastasis.[8] However in both no significant association of cyclin D1 positivity with tumor invasion, lymph node metastasis or differentiation was observed.[8,11]

p16 is a cyclin dependent kinase inhibitor that regulates cell cycle progression. Alterations of p16 occur by multiple mechanisms, including mutation, loss of heterozygosity (LOH) and promoter hypermethylation.[16] Immunohistochemical staining is useful for the detection and localization of aberrant p16 expressionin tumor samples.[8] Different studies showed variable loss of p16 expression in ESCC.

In the present study, 73.3 % of cases showed negativity for p16. Takeuchi and co-workers detected loss of p16 in expression 50%, Mathew and colleagues in 45% of ESCC, and Taghavi and colleagues in 56%.[8,12,17] In the present study the percentage of p16 negativity was higher than the other studies. Increased loss of p16 expression in the present study may be a consequence of various environmental and lifestyle factors specific to this region associated with an increased susceptibility to ESCC as mentioned above.

The study on association of p16 expression and the clinicopathological parameters showed that the percentage of tumors which were more than 3 cm (73.3%), tumors with invasion to the adventitia (82.4%), lymph node metastasis (68.8%) and tumors which were poorly differentiated (92.9%) were p16 negative. However, there was no significant statistical association in p16 expression with any of these parameters. Our findings are similar to several other studies.[8,18] There was significant association between the histological grading and p16 expression (p=0.012). There was a tendency for a decreased percentage of p16 negativity in poorly differentiated ESCC compared with moderately differentiated ESCC. There was no case of well differentiated ESCC, which were p16 negative. The association of p16 alterations with advanced-stage ESCC suggests that p16 alterations confer tumor cells with invasiveness and aggressiveness. From the therapeutic point of view this is important as cells lacking p16 are resistant to DNA damage-induced growth arrest compared with cells that retain p16.[19]

In the present study, 70% of the patients were both positive for cyclin D1 and negative for p16. In another study, 71% of the patients were positive for cyclin D1 and negative for p16.[8] The mechanism underlying the correlation between cyclin Dl overexpression and loss of pl6 expression has not been identified, but these results suggest that accumulation of many kinds of gene alterations occurs during oncogenesis and tumor progression. Furthermore, the p16 and cyclin D1 alterations may be linked because these cell cycle regulators are associated with CDK4-mediated phosphorylation of pRb.[8]

A small sample size was the major limitation of the study and a larger cohort over a longer duration is required to arrive at a conclusive result. However the present study attempts to analyze the role of p16 and cyclin D1 in ESCC in a region, where limited data is available regarding ESCC.

In conclusion, we report that alterations of Cyclin D1 and p16 play an important role in ESCC. The deregulated proteins confer an aggressive behavior to the tumor cells. Loss of p16 expression was associated with poor differentiation however cyclin Dl overexpression was independent of clinicopathological factors in this study.

CONFLICT OF INTEREST

The authors declare no conflict of interest related to this work.

REFERENCES

1. Parkin M D, Pisani P, Ferlay J. Estimates of the worldwide incidence of 25 major cancers in 1990. *Int J Cancer* 1999;**80**:827-41.
2. Enzinger P C, Mayer R J. Esophageal cancer. *N Engl J Med* 2003;**349**:2241-52.
3. Lehrbach D M, Nita M E, Cecconello I, Clinical Genomics of Esophageal Cancer Group. Molecular aspects of esophageal squamous cell carcinoma carcinogenesis. *Arq Gastroenterol* 2003;**40**:256-61.
4. Phukan R K, Ali M S, Chetia C K, Mahanta J. Betel nut and tobacco chewing; potential risk factors of cancer of oesophagus in Assam, India. *Br J Cancer* 2001;**85**:661–7.
5. Lin D C, Du X L, Wang M R. Protein alterations in ESCC and clinical implications: a review. *Dis Esophagus* 2009;**22**:9-20.
6. Metzger R, Shneider P M, Warnecke-Eberz U, Brabender J, Holscher A H. Molecular biology of esophageal cancer. *Onkologie* 2004;**27**:200-6.
7. Sobin L, Gospodarowicz M, Wittekind C, editors. UICC TNM classification of malignant tumors. 7th ed. New Jersey: Wiley Blackwell;2009.p 68-70.
8. Takeuchi H, Ozawa S, Ando N, Shih CH, Koyanagi K, Ueda M et al. Altered p16/ MTS1/ CDKN2 and cyclin D1/ PRAD-1 gene expression is associated with the prognosis of squamous cell carcinoma of the esophagus. *Clin Cancer Res* 1997;**3**:2229-36.
9. Park J C, Moon C. Gene therapy for esophageal cancer. *Cancer Therapy* 2008;**6**:35-46.
10. Chetty R, Chetty S. Cyclin D1 and retinoblastoma protein expression in oesophageal squamous cell carcinoma. *J Clin Pathol Mol Pathol* 1997;**50**:257-60.
11. Lin Y C, Wu M Y, Li D R, Wu X Y, Zheng R M. Prognostic and clinicopathological features of E-cadherin, a-catenin, b-catenin, g-catenin and cyclin D1 expression in human esophageal squamous cell carcinoma. *World J Gastroenterol* 2004;**10**:3235-9.
12. Mathew R, Arora S, Khanna R, Mathur M, Shukla N K, Ralhan R. Alterations in p53 and pRb pathways and their prognostic significance in oesophageal cancer. *Eur J Cancer* 2002;**38**:832-41.
13. Phukan R K, Chetia C K, Ali M S, Mahanta J. Role of Dietary Habits in the Development of Esophageal Cancer in Assam, the North-Eastern Region of India. *Nutr Cancer* 2001;**39**:204 -9.
14. Lee C H, Lee J M, Wu D C, Hsu H K, Kao E L, Huag H L et al. Independent and combined effects of alcohol intake, tobacco smoking and betel quid chewing on the risk of esophageal cancer in Taiwan. *Int J Cancer* 2005;**113**:475-82.
15. Kumpawat K, Chatterjee A. The usefulness of cytogenetic parameters, level of p53 protein and endogenous glutathione as intermediate end-points in raw betel-nut genotoxicity. *Hum Exp Toxicol* 2003;**22**:363-71.
16. Paulson T G, Galipeau P C, Xu L, Kissel H D, Li X, Blount P L et al. p16 mutation spectrum in the premalignant condition Barrett's esophagus. *PLoS One* 2008;**3**:e3809.
17. Taghavi N, Biramijamal F, Sotoudeh M, Khademi H, Malekzadeh R, Moaven O et al. p16INK4a hypermethylation and p53, p16 and MDM2 protein expression in esophageal squamous cell carcinoma. *BMC Cancer* 2010;**10**:138.
18. Sturm I, Petrowsky H, Volz R, Lorenz M, Radetzki S, Hillebrand T et al. analysis of p53/BAX/p16ink4a/CDKN2 in esophageal squamous cell carcinoma: high BAX and p16ink4a/CDKN2 identifies patients with good prognosis. *J Clin Oncol* 2001;**19**:2272-81.
19. Shapiro G I, Edwards C D, Ewen M E, Rollins B J. p16INK4A participation in a G1 arrest checkpoint in response to DNA damage. *Mol Cell Biol* 1998;**18**:378-87.